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SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction

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ABSTRACT **NOTE: Stem cell transplantation at the time of acute myocardial infarction (AMI) improves cardiac function. Whether the improved cardiac function results from regeneration of cardiac myocytes, modulation of remodeling, or preservation of injured tissue through paracrine mechanisms is actively debated. Because no specific stem cell population has been shown to be optimal, we investigated whether the benefit of stem cell transplantation could be attributed to a trophic effect on injured myocardium. Mesenchymal stem cells secrete SDF-1 and the interaction of SDF-1 with its receptor, CXCR4, increases survival of progenitor cells. Therefore, we compared the effects of MSC and MSC engineered to overexpress SDF-1 on cardiac function after AMI. Tail vein infusion of syngeneic MSC and MSC:SDF-1 1 day after AMI in the Lewis rat led to improved cardiac function by echocardiography by 70.7% and 238.8%, respectively, compared with saline controls 5 wk later. The beneficial effects of MSC and MSC:SDF-1 transplantation were mediated primarily through preservation, not regeneration of cardiac myocytes within the infarct zone. The direct effect of SDF-1 on cardiac myocytes was due to the observation that, between 24 and 48 h after AMI, SDF-1-expressing MSC increased cardiac myocyte survival, vascular density (18.2±4.0 *vs.* 7.6±2.3 vessels/mm^2, $P<0.01$; SDF-1:MSC *vs.* MSC), and cardiac myosin-positive area (MSC: 49.5%; mSC:SDF-1: 162.1%) within the infarct zone. There was no evidence of cardiac regeneration by the infused MSC or endogenous cardiac stem cells based on lack of evidence for cardiac myocytes being derived from replicating cells. These results indicate that stem cell transplantation may have significant beneficial effects on injured organ function independent of tissue regeneration and identify SDF-1:CXCR4 binding as a novel target for myocardial preservation.—Zhang, M., Mal, N., Kiedrowski, M., Chacko, M., Askari, A. T., Popovic, Z. B., Koc, O. N., Penn, M. S. SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. *FASEB J.* 21, 000–000 (2007)**

Key Words: HSC · cardiac regeneration · transplantation · SDF-1-based therapy

THE TRANSPLANTATION OF multiple stem cell types at the time of myocardial infarction has been shown to improve left ventricular perfusion and/or function in preclinical and clinical studies (1–6). While this strategy holds great promise for the prevention and treatment of congestive heart failure, the underlying mechanisms facilitating the improvement remain unclear. One possibility is that the transplanted stem cells regenerate myocardial tissue by differentiating into cardiac myocytes, endothelial cells, and smooth muscle cells (1, 7). Another less explored possibility is that the introduction of stem cells into the myocardium at the time of acute myocardial infarction (AMI) supports the injured tissue through as yet undefined trophic effects leading to preservation of cardiac myocytes and improved cardiac function. If trophic effects of stem cells prove important in improving cardiac function, the ability to harness or augment these effects may be possible through gene transfer strategies (8–10).

CXCR4 is the cell surface receptor for SDF-1, and is expressed on early hematopoietic stem cells (HSC) and endothelial progenitor cells (11–13). We recently demonstrated that stromal cell-derived factor-1 (SDF-1 or CXCL12) is expressed by the heart immediately after an AMI. In addition, re-establishing SDF-1 expression at a time remote from MI can restore stem cell homing to damaged cardiac tissue (14). Unfortunately, emerging data indicate that these cell types do not differentiate into cardiac myocytes (15, 16). While the expression of SDF-1 results in homing of HSC and endothelial progenitor cells to the injured myocardium (14, 17), evidence suggests that SDF-1 can have additional non-

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stem cell-recruiting effects, including increasing stem cell survival (18).

Recently, SDF-1 has been shown to have growth and survival benefits in CXCR4-expressing MSC. MSC normally express SDF-1; therefore, in an attempt to define the trophic effects of MSC stem cell infusion through SDF-1, we generated MSC that overexpressed SDF-1. We then compared the effects of saline-, MSC-, and SDF-1-overexpressing MSC on MSC survival, cardiac myocyte survival and regeneration, and cardiac function. Our results demonstrate a significant role for non-stem cell homing trophic effects of SDF-1 on injured myocardium.

MATERIALS AND METHODS

LAD ligation

All animal protocols were approved by the Animal Research Committee and all animals were housed in the AAALAC animal facility of the Cleveland Clinic. Ligation of the left anterior descending artery in Lewis rats was performed as described previously (9, 14). Briefly, animals were anesthetized with i.p. ketamine and xylazine, intubated, and ventilated with room air at 75 breaths per minute using a pressure-cycled rodent ventilator (RSP1002, Kent Scientific Corp., Torrington, CT, USA). An anterior wall myocardial infarction was induced by direct ligation of the left anterior descending (LAD) artery with the aid of a surgical microscope (M500, Leica™ Microsystems, Bannockburn, IL, USA).

Cell preparation and delivery

Rat bone marrow was isolated by flushing Lewis rat femurs with 0.6 ml DMEM (Gibco, Invitrogen, Carlsbad, CA, USA). Clumps of bone marrow were gently minced with a 20 gauge needle. Cells were separated by Percoll density gradient. The cells were centrifuged for 10 min at 260 *g* and washed with three changes of PBS with 100 U/ml penicillin 100 *g*/ml streptomycin (Invitrogen). The washed cells were resuspended and plated in DMEM-LG (Gibco, Invitrogen) with 10% FBS and 1% antibiotic and antimycotic solutions (Gibco, Invitrogen), then incubated at 37°C. Nonadherent cells were removed by replacing the medium after 3 days. Cultures were refed every 3–4 days. Once cultures became 70% confluent, adherent cells were detached after incubation with 0.05% trypsin and 2 mM EDTA (Invitrogen) for 5 min and subsequently passaged. Preceding experiments utilizing MSC, the cultures were simultaneously depleted of $CD45^+$, $CD34^+$ cells by negative selection using 10 μl per 10^6 cells of each of the after primary PE-conjugated antibodies: mouse anti-rat CD45 (BD Biosciences, San Diego, CA, USA) and mouse anti-CD34 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). PE-positive cells were negatively selected using the EasySep PE selection kit according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, B.C., Canada).

Confluent cells were passaged and plated out at 1:2 to 1:3 dilutions until passage 11. Cells were assayed for their ability to differentiate into the adipogenic, chondrogenic, and osteogenic lineages. Cells were maintained in differentiation media for 2 to 3 wk. Differentiation was validated by staining the cells with Oil Red (adipogenic lineage), alcian blue (chondrogenic lineage), or alkaline phosphatase (osteogenic lineage). Cells were induced to overexpress SDF-1 by transfection with a plasmid construct that encoded SDF-1 (14) and antibiotic selection to select for a population of cells that was stably transfected. Two million labeled cells (cardiac fibroblasts, MSC, or SDF-1-expressing MSC) suspended in 200 ml of PBS or 200 ml of PBS alone were infused *via* tail vein injection 24 h after myocardial infarction.

Cardiac fibroblast preparation

Lewis rat cardiac fibroblast were prepared as described previously (14). Briefly, the ventricles of three to five hearts from adult male Lewis rats were minced, pooled, and placed in 100 U/ml of type XI collagenase (Sigma, St. Louis, MO, USA) and 0.1% trypsin (Life Technologies, Inc., Gaithersburg, MD, USA) digestion solution at 37°C for 10 min in a shaking water bath. Fibroblasts were isolated subsequently from enriched fractions and suspended in Ham's modified Dulbecco's medium (Media Core Facility, CCF) supplemented with heat-inactivated 10% FBS and 1% antibiotic and antimycotic (Gibco, Invitrogen) and plated onto T75 tissue culture flasks (Falcon, Oxnard, CA, USA; BD PharMingen, San Diego, CA, USA). Nonadherent cells were removed after 2 h and discarded. Cells were fed with fresh medium three times per week, split 1:3 when confluent, and used for subsequent experiments.

BrdU labeling of cells *in vivo*

MSC in vitro prior to cell transplantation

MSC (passage 6) were stably transfected with rat SDF-1 expression vector or pcDNA3.1 (control vector) as described previously (14). Two days before infusion, the cells were freshly plated in a 1:3 ratio and incubated in complete medium with 10 μM BrdU (5-bromo 2-deoxyuridine) to label cells in the S phase of the cell cycle during the 48 h period prior to harvest for cell transplantation.

Cell in vivo after cell transplantation

In those studies in which proliferating cells *in vivo* were labeled, BrdU (50 mg/kg) was injected i.p. every 12 h for 14 days beginning the day after cell transplantation.

GFP labeling of cells

We used a VSV-G pseudotyped lentivirus-expressing EGFP with a nuclear localizing sequence or SDF-1 driven by the CMV promoter. The lentivirus was made using four plasmid vector system by the Viral Core at the Cleveland Clinic. The MSC were transduced twice for 8 h with purified lentivirus in the presence of 8 μg/ml of polybrene at a multiplicity of infection (MOI) of 30. The media was changed 72 h post-transfection and replaced with regular media containing zeocin (EGFP) or zeocin and blasticidin (hSDF1 and EGFP). Thus, only cells that have incorporated the viral genome, including the zeocin and/or blasticidin resistance gene, survived.

Real-time PCR

RT-PCR was performed after isolation of RNA from 6 million cells by using an Rneasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). The reaction mixture contained SYBR green PCR

master mix (Applied Biosystems), each primer at 300 nM, and 10 μl of cDNA. After activation of the AmpliTaq Gold (Applied Biosystems) for 10 min at 95°C, we carried out 45 cycles, each consisting of 15 s at 95°C followed by 1 min at 60°C. The dissociation curve for each amplification was analyzed to confirm that there were no nonspecific PCR products. CXCR4 primer sequences: forward: ATCATCTC-CAAGCTGTCACACTCC; reverse: GTGATGGAGATCCACT-TGTGCAC.

Immunostaining

Animals were sacrificed 96 h or 5 wk after myocardial infarction. Tissues were fixed in formalin and embedded in paraffin blocks according to established protocols. Antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) and heat at 95°C for 5 min. The buffer was replaced with fresh buffer and reheated for an additional 5 min, then cooled for ~20 min. The slides were washed in deionized water three times for 2 min each. Specimens were then incubated with 1% normal blocking serum in PBS for 60 min to suppress nonspecific binding of IgG. Slides were then incubated for 60 min with the mouse anti-BrdU primary antibody (BD Biosciences, San Jose, CA, USA). Optimal antibody concentration was determined by titration. Slides were then washed with phosphate-buffered saline (PBS) and incubated for 45 min, with FITC-conjugated secondary antibody (Santa Cruz Biotechnology) diluted to 1.5 μg/ml in PBS with serum in a dark chamber. After washing extensively with PBS, coverslips were mounted with aqueous mounting medium (Vectashield Mounting Medium with DAPI, H-1200; Vector Laboratories, Burlingame, CA, USA).

Confocal immunofluorescence microscopy

Tissues were analyzed using an upright spectral laser scanning confocal microscope (Model TCS-SP; Leica Microsystems, Heidelberg, Germany) equipped with blue argon (for DAPI), green argon (for Alexa Fluor 488), and red krypton (for Alexa Fluor 594) laser. Data were collected by sequential excitation to minimize "bleed-through." Image processing, analysis, and the extent of colocalization were evaluated using Leica Confocal software. Optical sectioning was averaged over four frames and the image size was set at 1024 × 1024 pixels. There were no digital adjustments made to the images.

Histological studies

Myocyte cross-sectional dimensions in the peri-infarct zone were measured from hematoxylin and eosin (H&E) -stained slides. Images were obtained by a blinded observer using Leica DMR upright microscope (Leica Co., Heidelberg, Germany) with cooled CCD camera (Q-imaging Retiga Ex, British Columbia, Canada). Morphometric analysis of H&E-stained tissues was performed using ImagePro Plus software at a magnification of 200×. Cross-sectional diameters of five randomly chosen cardiac myocytes were measured as described (19) in each of eight randomly chosen fields per animal by two observers blinded to the identity of the animal from which the images were obtained.

Flow cytometric analysis

MSC cultures were prepared by trypsin/EDTA digest. Cells were washed twice with cold (1×) D-PBS, resuspended in 1× binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) at a concentration of 1×10^6 cells/ml, and 100 μl (1×10^5) cells were transferred to a 5 ml tube. Single-cell suspensions were then incubated with either 1 μl of Annexin V-PE-Cy5 (Abcam, Cambridge, MA, USA) or 5 μl propidium iodide (PI) (BD Biosciences, San Diego, CA, USA) or isotype-matched control antibody. The cells were vortexed gently and incubated at room temperature for 15 min in the dark. Then 400 ml of 1× binding buffer was added to each tube and the samples data were acquired by a Guava EasyCyte flowcytometer (Guava Technologies Hayward, CA, USA) and analyzed with FlowJo (Tree Star, Inc., Ashland, OR, USA) flow cytometric analysis programs within 1 h.

TUNEL assay for assessment of apoptotic cell death

TUNEL was performed to detect apoptotic nuclei using terminal deoxynucleotidyl transferase (TdT) -mediated *in situ* fluorescein-conjugated, dUTP nick end-labeling technique according to the manufacturer's protocol (Roche, Indianapolis, IN, USA). The sections were incubated again with mouse monoclonal antibody (Chemicon International, Inc., Temecula, CA, USA) -recognizing cardiac ventricular myosin heavy chain α/β to specifically recognize apoptotic cardiomyocytes. The fluorescence staining was viewed with a confocal laser scanning microscope. The number of apoptotic cells was counted and expressed as a percentage of total myocyte population.

Western protocol

Cell extracts were prepared in 4× reducing Lamellae buffer (200 mM Tris HCl (pH 6.8), 8% SDS, 0.1% bromphenol blue, 40% glycerol). Sodium dodecyl sulfate (SDS) gels were prepared according to established protocols. Proteins were separated in a 10% SDS-polyacrylamide gel. The blotting membrane was placed in 5% milk in 1× TBST (Tris base, 2.42 g; NaCl, 8 g; 1M HCl, 3.8 ml with pH to 7.5; water, 1 L, Tween 20, 2 ml) for 1 h, then probed with primary antibody (1:1000 in 5% milk in 1× TBST) against phosphorylated *Akt* (Santa Cruz Biotechnology), followed by incubation with the peroxidase-conjugated anti-mouse secondary antibody (1:5000 in 1× TBST). Chemiluminescence (Amersham Biosciences UK Limited, Buckinghamshire, UK) was used for visualization.

Antibodies implemented in these studies

Primary antibodies

Mouse anti-myosin ventricular heavy chain alpha/beta monoclonal antibody (Chemicon International); mouse monoclonal anti-alpha-sarcomeric actin IgM (Sigma); mouse antitroponin I monoclonal IgG2b antibody (Chemicon International); rabbit anti-GATA 4 polyclonal IgG antibody (Santa Cruz Biotechnology); goat polyclonal anti-Nkx-2.5 IgG antibody (Santa Cruz Biotechnology); rabbit polyclonal anti-MEF-2 IgG antibody (Santa Cruz Biotechnology); mouse monoclonal anti-alpha smooth muscle actin-Cy3 conjugated antibody (Sigma); rabbit polyclonal anti-human von Willebrand factor; rabbit anticonnexin-43 polyclonal IgG antibody (Santa Cruz Biotechnology); rabbit anticonnexin 45 polyclonal IgG antibody (Santa Cruz Biotechnology); goat polyclonal anticonnexin-40 IgG antibody (Santa Cruz Biotechnology); mouse IgG1 monoclonal anti-Akt1 antibody (Cell Signaling Technology); mouse monoclonal anti-phospho-Akt (Ser-473 IgG2b antibody (Cell Signaling Technology); rabbit polyclonal anti-CXCR4 IgG (Abcam); rat monoclonal anti-BrdU-FITC conjugated (Abcam).

Secondary antibodies

Goat anti-mouse IgG Alexa Fluor 488 (Molecular Probes, Carlsbad, CA, USA); goat anti-mouse IgG Alexa Fluor 594 (Molecular Probes); donkey anti-rabbit IgG Alexa Fluor 488 (Molecular Probes); donkey anti-rabbit IgG Alexa Fluor 594 (Molecular Probes); goat polyclonal IgG anti-fluorescein antibody (Molecular Probes); donkey anti-goat IgG Alexa Fluor 488 antibody (Molecular Probes); donkey anti-goat IgG antibody Alexa Fluor 594 (Molecular Probes); goat anti-mouse IgM Alexa Fluor 488 (Molecular Probes).

Echocardiography

2-Dimensional echocardiography was performed 2 and 5 wk after LAD ligation and MSC transplantation using a 15 MHz linear array transducer interfaced with a Sequoia C256 and GE Vision 7 as described previously (9, 14). LV dimensions and wall thickness were quantified by digitally recorded 2-dimensional clips and M-mode images in a short axis view from the mid-LV just below the papillary muscles to allow for consistent measurements from the same anatomical location in different rats. The ultrasonographer was blinded to treatment group. Measurements were made by two independent blinded observers off-line using ProSolv echocardiography software. Measurements in each animal were made six times from three of five randomly chosen M-mode clips recorded by an observer blinded to the treatment arm. Shortening fraction was calculated from the M-mode recordings. Shortening fraction (%) = (LVEDD−LVESD)/LVEDD × 100, where LVEDD = left ventricular end diastolic dimension and LVESD = left ventricular end systolic dimension.

Statistical analyses

Data are presented as mean ± SD. Comparisons between groups were done by unpaired Student's *t* test (vascular density and cell engraftment data) or by ANOVA with Bonferroni correction (echocardiographic and cardiac myosin area data) for multiple comparisons where cell treatment was compared with saline controls, then significant differences between MSC and SDF-1:MSC were determined.

RESULTS

Characterization of engineered MSC

We generated MSC that were stably transfected with an SDF-1 expression vector driven by the CMV promoter (14). The MSC used in our studies expressed CXCR4 by RT-PCR, Western blot (**Fig. 1*a***), and immunohistochemistry (Fig. 1*b*). The population of stably transfected MSC used in our studies expressed 5.29 ± 1.25-fold greater SDF-1 mRNA than MSC transfected with the control construct. Transfection with SDF-1 expression vector did not change CXCR4 expression (0.81±0.24, relative CXCR4 mRNA and 1.19±0.18, relative CXCR4 protein expression in SDF-1 overexpressing to control MSC, n=4 independent cell preparations and 3 analyses per cell preparation). Over a 24 h period in culture, SDF-1 overexpressing MSC secreted significantly greater amounts of SDF-1 into the media than MSC transfected with control vector (Fig. 1*c*). No significant release of SDF-1 was observed in parallel cultures of cardiac fibroblasts. Consistent with SDF-1 inducing up-regulation of prosurvival signaling, as seen in progenitor cells (18, 20, 21), the MSC that overexpressed SDF-1 had greater phosphorylated Akt than control cells (% phos-Akt to Akt: 20.2%±6.7% *vs.* 5.2%±2.1%, SDF-1-expressing *vs.* control MSC, P=0.005, n=4 independent cell preparations and 3 analyses per cell preparation, Fig. 1*d*).

Effects of SDF-1 on MSC survival during hypoxia

To determine whether SDF-1:CXCR4 binding could improve MSC survival, we cultured control MSC and SDF-1:MSC under hypoxic conditions (1% oxygen) for

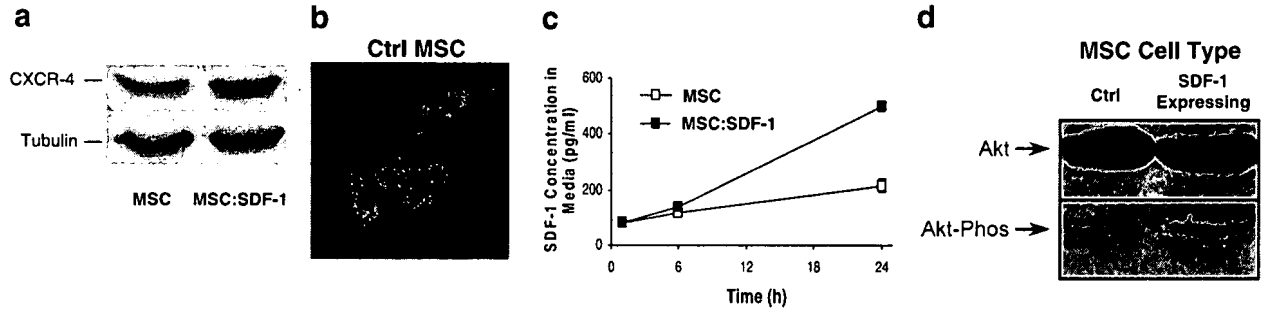

Figure 1. *a)* Western blot for CXCR4 and tubulin as a loading control in control MSC and SDF-1:MSC and *b)* immunofluorescence staining for CXCR4 in control MSC. *c)* Ten thousand control and SDF-1-expressing MSC were plated separately per well in a 12-well plate in serum-free DMEM. A 100 μl of media was obtained 1, 6, and 24 h later. SDF-1 levels in the media were quantified using ELISA (R&D Systems, Minneapolis, MN, USA). An equal cell number was verified by quantifying total protein per cell layer at the end of the experiment. Data is expressed as picograms of SDF-1 per ml of total media. Experiments were performed in triplicate. Data represent mean ± SD. *d)* Western blot analysis for Akt and phosphorylated Akt in control and SDF-1-expressing MSC. Western blots were performed with 50 μg of total cell protein separated on a 10% SDS-PAGE gel.

72 h and quantified evidence of cell injury using FACS. The data in **Fig. 2*a*** demonstrate that >25% of control MSC grown under hypoxic conditions express Annexin V compared with <10% of SDF-1:MSC. Similar results were observed when the percentage of propidium iodide-positive cells, a marker of cell death, was quantified (data not shown).

We assessed whether similar results would be observed *in vivo* after an acute anterior wall myocardial infarction induced by direct LAD ligation. Twenty-four hours later, 2 million syngeneic MSC stably transfected with empty plasmid or plasmid encoding SDF-1 were infused by tail vein injection. In both groups, the MSC were labeled with a Lentivirus encoding GFP. Control rats received an intravenous infusion of saline.

Seventy-two hours, 3 and 5 wk after infusion of cells, the number of MSC (GFP+cells) in the heart was significantly increased by the overexpression of SDF-1 (Fig. 2*b*), although in both groups there was a significant decrease in the number of engrafted MSC at 5 wk compared with 72 h after treatment (Fig. 2*c*). Significant homing or engraftment of 2 million infused cardiac fibroblasts was not observed (4 days: 3.6±2.7 cells/mm^2 and 5 wk: 2.9±2.1 cells/mm^2).

Effect of SDF-1 overexpression on ischemic myocardium

CXCR4 expression in the infarct zone is demonstrated as early as 24 h after AMI (**Fig. 3*a***, 24 h). Cardiac myocytes in the infarct border zone begin to express CXCR4 as early as 48 h after AMI Fig. 3*a* (24–48 h), and the level of cardiac myocyte CXCR4 expression within the infarct border zone increases for up through 96 h after AMI.

Overexpression of SDF-1 within the infarct zone *via* the infusion of SDF-1-expressing MSC led to a significant decrease in the number of TUNEL-positive cardiac myocyte nuclei (~67% decrease) (Fig. 3*b–d*). This decrease in cardiac myocyte apoptosis was accompanied by a significant increase in the area of surviving bundles of cardiac myocytes within the infarct zone compared with saline controls (Fig. 3*e, f*). The increase in cardiac myocyte area was not due to cardiac hypertrophy since the average cardiac myocyte diameter

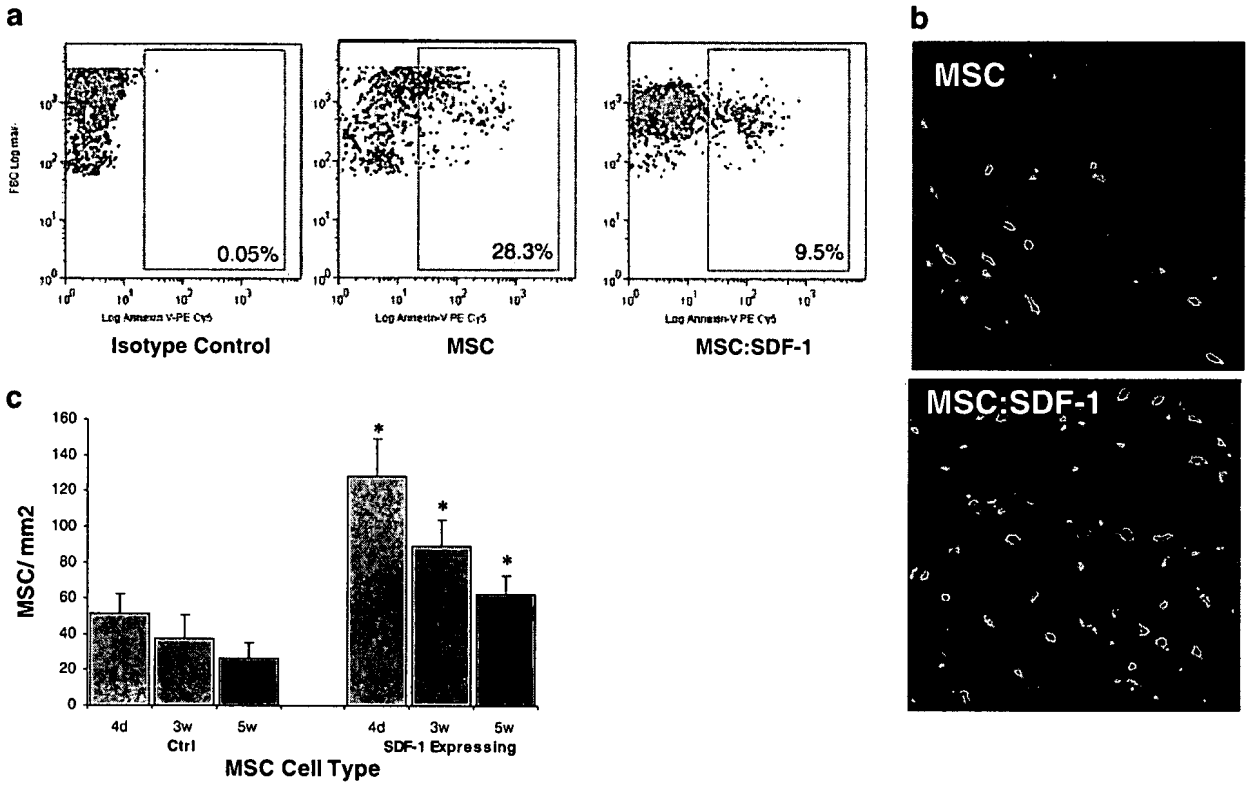

Figure 2. *a)* Representative FACS analyses for Annexin V-positive cells in cultures of MSC- or SDF-1-expressing cells after 72 h of being cultured under hypoxic condition (0.1% oxygen) in serum-deprived culture medium (1% FBS). *b)* Representative immunofluorescent staining for GFP (FITC, green) and nuclei (DAPI, blue) within the infarct zone 96 h after LAD ligation from rats that received 2 million control (left) or SDF-1-overexpressing MSC (right) 24 h after LAD ligation. *c)* Number of MSC per square millimeter within the infarct zone at 4 days (d) and 3 and 5 wk (w) after LAD ligation. Animals received 2 million control or SDF-1-overexpressing MSC 24 h after LAD ligation. MSC per square millimeter was quantified after immunofluorescent staining for GFP. Two independent observers blinded to treatment group quantified the number of GFP- and DAPI-positive nuclei in the infarct zone in 10 random fields from 5 different sections (total 50 fields) obtained from the mid-left ventricle. Data represent mean + SD. $*P < 0.01$ compared with Ctrl MSC infusion.

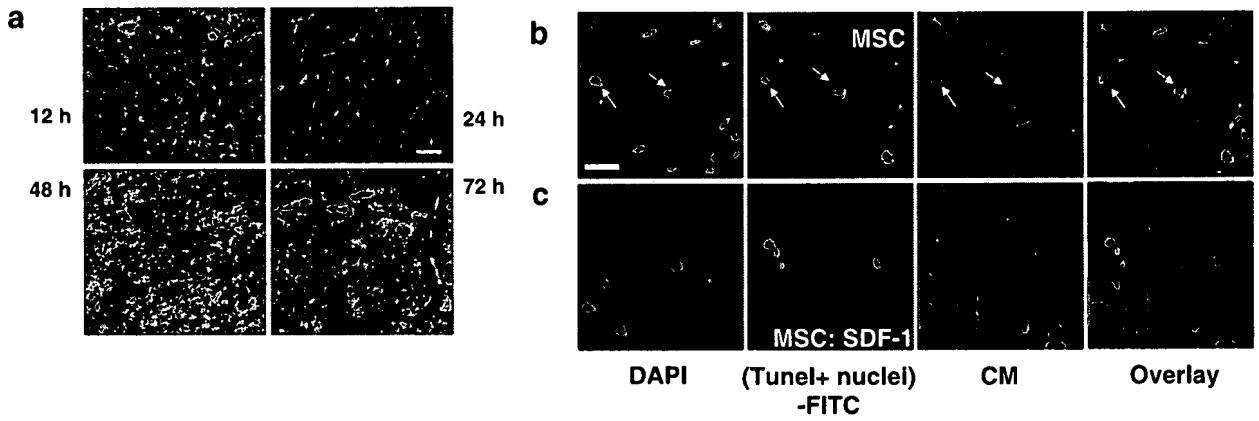

Figure 3. *a)* Confocal image of representative immunofluorescent staining for CXCR4 (Alexa Fluor 488, green) and Troponin I (Alexa Fluor 594, red) in the infarct border zone 12–72 h after LAD ligation. *b, c)* Confocal image of representative immunofluorescent staining for left) cardiac myosin (Alexa Fluor 594, red), center) TUNEL (Alexa Fluor 488, green), and right) merged image from an animal 96 h after LAD ligation and 72 h after infusion of *b)* control and *c)* SDF-1-expressing MSC. Arrows identify the same nuclei in each picture of a given series. *d)* Number of TUNEL-positive nuclei in the infarct border zone 96 h after LAD ligation in animals that received control or SDF-1 overexpressing MSC 24 h after AMI. Two independent observers blinded to treatment group quantified the number of TUNEL-positive nuclei in 1000 nuclei within 4–5 cells from the infarct border zone from 5 different sections (total 5000 nuclei total) obtained from the mid-left ventricle. Data represent the mean percent TUNEL-positive cells ± SD. $*P < 0.0001$ compared with Ctrl MSC infusion. *e)* Percent area positive for cardiac myosin within the infarct zone 5 wk after LAD ligation in animals that received saline-, control, or SDF-1-overexpressing MSC 24 h after AMI. Percent cardiac myosin-positive area was obtained by segmenting the image based on grey scale value using NIH Image by an observer blinded to treatment group. Five sections per animal were quantified. Data represent mean ± SD. $^{\&}P < 0.01$ and $*P < 0.0001$ compared with saline infusion. *f)* Representative sections obtained 5 wk after AMI stained for cardiac myosin (FITC, green) from animals that received saline-, control, or SDF-1-overexpressing MSC.

within the infarct border zone was the same in all groups (saline, 24.2±7.1 μm; mSC, 24.4±2.4 μm; mSC: SDF-1, 24.0±3.9 μm). The cardiac myocytes within the infarct zone at this time point were not GFP positive; therefore, they were not regenerated from the engrafted MSC.

Effects of SDF-1 overexpression on cardiac remodeling and function

Left ventricular function and dimensions were quantified 14 and 35 days after LAD ligation in animals that received saline, cardiac fibroblasts, control, or SDF-1-overexpressing MSC 1 day after LAD ligation. A statistically significant attenuation of LV dilation and improvement in shortening fraction was demonstrated with MSC infusion compared with saline controls (**Fig. 4*a*** and ***b***, respectively). In those animals treated with control and SDF-1-expressing MSC, shortening fraction was increased by 74% and 246% (at 5 wk: saline *vs.* MSC *vs.* SDF-1:MSC fractional shortening: 10.9% ± 1.0% *vs.* 17.6% ± 3.0% *vs.* 34.9% ± 8.5%), respectively, compared with saline controls. Similarly, LV dimensions were significantly decreased in those animals that received SDF-1:MSC compared with control MSC or saline (at 5 wk: saline *vs.* MSC *vs.* SDF-1:MSC LVEDD: 10.9±1.0 *vs.* 10.0±0.9 7.6±1.0 and LVESD: 9.7±1.8 *vs.* 8.2±1.2 *vs.* 5.0±2.4). No significant difference was observed between saline infusion and cardiac fibroblast infusion.

Immunofluorescence using antibody for vWF was used to identify and quantify the vascular density within the infarct zone after each treatment. A significant increase in the number of capillaries and small arterioles was observed in those animals that received SDF-1-overexpressing MSC (18.2±4.0 *vs.* 7.6±2.3 vessels/mm^2, $P<0.01$, **Fig. 5*a***). This observation is consistent with earlier studies that have demonstrated that local SDF-1 expression leads to homing of endothelial progenitor cells (13, 14).

Cardiac myocyte regeneration *vs.* preservation

The data in Fig. 3 demonstrate that MSC and, to a greater extent, SDF-1-expressing MSC, increase the area and number of cardiac myocytes within the infarct zone. While the data in Figs. 1–3 support the concept that this increase is due to cardiac preservation, we wanted to determine the extent to which either the injected MSC or the endogenous cardiac stem cells participated in cardiac myocyte regeneration.

To determine the fate of the engrafted MSC, we stained sections of myocardial tissue from each group for markers of cardiac myocytes (cardiac myosin, troponin I, GATA4, and connexin 43), smooth muscle cells (SMC α-actin and connexin 45), myofibroblasts (SMC α-actin and vimentin), and endothelial cells (vWF and connexin 40). GFP- or BrdU-labeled MSC were not vWF and were rarely (<2%) cardiac myosin positive, suggesting that with or without SDF-1 transfection, MSC appear not to differentiate into cardiac myocytes. We observed that a significant percentage of the GFP-labeled cells engrafted into the myocardium were SMC α-actin-positive whether they were infused as

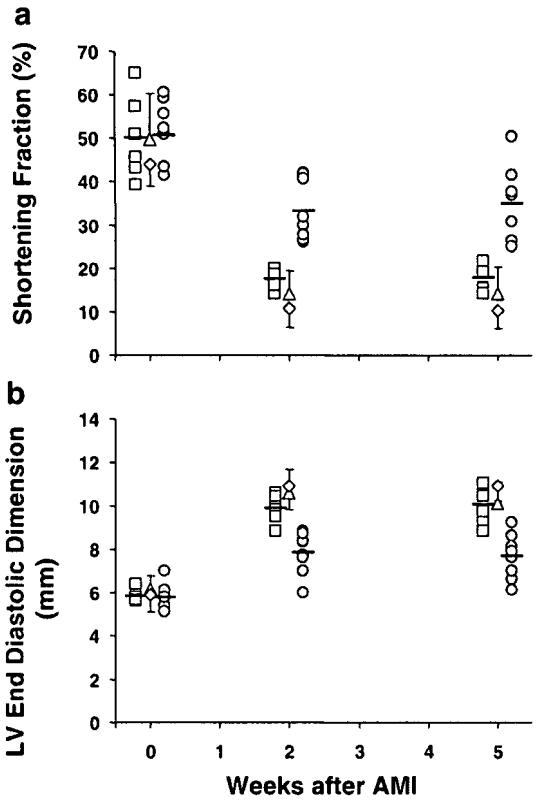

Figure 4. *a)* Cardiac function and *b)* left ventricular size as quantified by the echocardiographic parameters shortening fraction and left ventricular end diastolic dimension (LVEDD), respectively. 2-Dimensional and M-mode echocardiography were performed at baseline 2 and 5 wk after LAD ligation in animals that received saline (diamond, $n=7$) or 1 million cardiac fibroblasts (triangle, $n=5$), control MSC (open square, $n=6$), or SDF-1-overexpressing MSC (filled circle, $n=8$). For animals that received saline and cardiac fibroblasts, data represent mean ± SD. For animals that received MSC, individual data points are presented and the mean for that group is represented by a horizontal line. $^{\&}P < 0.01$ and $^{*}P < 0.0001$ compared with saline infusion.

control MSC or SDF-1:MSC (Fig. 5*b*); however, the percentage of the area of the infarct zone containing SMC α-actin cells was increased in the SDF-1:MSC-treated group (MSC *vs.* SDF-1:MSC: 9.4%±2.9% *vs.* 41.3%±1.0%, $P<0.001$). Immunofluorescence staining further demonstrated that a significant number of SMC α-actin-positive cells were also vimentin positive, suggesting that the MSC significantly increased the number of myofibroblasts in the infarct zone (22) and that the addition of SDF-1 further increased the percentage of the area of the infarct zone containing myofibroblasts (vimentin and SMC α-actin-positive cells; mSC *vs.* SDF-1:MSC: 7.2%±4.1% *vs.* 32.2%±8.0%, $P<0.001$).

We stained these sections for connexin 40, 43, and 45 to determine whether these SMC α-actin cells could be electrically coupled, and thus contribute to the improved cardiac function observed in animals that received SDF-1-expressing MSC. These studies demonstrated that the myofibroblasts were connexin 45 positive, but not connexin 40 or 43 positive (data not shown).

To determine whether cardiac stem cells led to the regeneration of cardiac myocytes, we repeated our studies using GFP-labeled MSC and GFP-labeled SDF-1 overexpressing MSC and administered BrdU to the animals twice daily beginning the day after cell transplantation. We hypothesized that if cardiac stem cells differentiate into cardiac myocytes after LAD ligation and MSC infusion, they would proliferate prior to migration and/or differentiation. Therefore, if there were no BrdU-positive cardiac myocytes, we could exclude a role for cardiac stem cells in cardiac myocyte regeneration in this model.

The data in **Fig. 6** show representative images from saline-, MSC-, and SDF-1:MSC-treated animals double stained for BrdU and cardiac myosin. There are more BrdU-positive cells in the SDF-1:MSC-treated animals than in MSC- and saline-treated animals; however, there is no evidence that mature cardiac myocytes are BrdU positive in any of our treatment groups. These data are consistent with the hypothesis that the increased cardiac myocyte areas we observe in response to MSC and MSC that overexpress SDF-1 are not due to regeneration from cardiac stem cells or bone marrow-derived stem cells.

To determine whether the engrafted MSC proliferated within the myocardial tissue, we double stained BrdU and GFP tissue sections from saline-, MSC-, and SDF-1-expressing overexpressing MSC-treated animals. We observed significant MSC proliferation with control and SDF-1 overexpressing MSC; however, the majority of BrdU-positive cells within the tissue sections were not derived from the infused MSC (data not shown).

DISCUSSION

The goal of stem cell-based therapies after AMI is to *1)* minimize myocardial cell death, *2)* optimize LV remodeling, and *3)* regenerate myocardial structures, including blood vessels and cardiac myocytes. Recent studies have suggested that stem cell engraftment into recently infarcted myocardium can lead to improved cardiac function. Whether this is guided by a cache of resident cardiac stem cells that replace damaged myocardium, bone marrow-derived stem cells that home to damaged myocardium, or exogenous cells infused intravenously after MI is not fully understood (23). Furthermore, the ability of hematopoietic stem cells to transdifferentiate into cardiac myocytes remains a matter of ongoing debate (1, 7) but appears unlikely given recent results (15, 16). Despite this uncertainty, it is clear that the introduction of a variety of stem cell types from varied sources can lead to improved cardiac function. These findings ultimately suggest that a naturally occurring albeit clinically inefficient cardiac reparative system seems to exist at some basal level that is potentially exploitable (14).

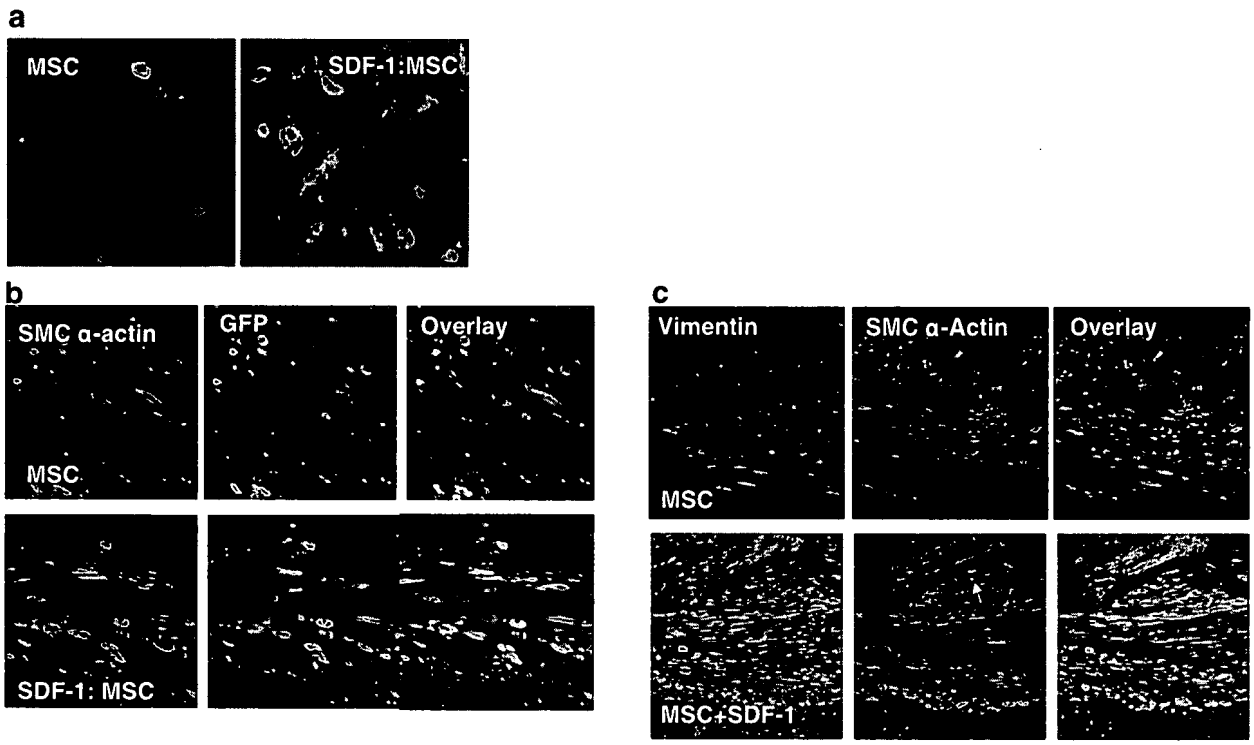

Figure 5. All representative images are from tissue 5 wk after AMI and infusion of 2 million of MSC or SDF-1:MSC 1 day after AMI. *a)* High-power image of immunofluorescent staining for smooth muscle cell α-actin (Cy3, red), cell nuclei (DAPI, blue), and vWF (FITC, green). *b)* Immunofluorescent staining for vimentin (FITC, green), smooth muscle cell α-actin (Cy3, red) from animals that received MSC (top) or SDF-1:MSC (bottom). *c)* Confocal image of staining for α-actin (Cy3, red), cell nuclei (DAPI, blue), and connexin 45 (FITC, green) from an animal that received SDF-1-expressing MSC low-power confocal image of immunofluorescent staining of connexin 45 (Alexa Fluor 488, green) and α-actin (Cy3, red).

Effects of SDF-1 in post-MI myocardial tissue

The goal of our study was to determine the potential role SDF-1 has in the reparative process and to determine whether overexpressing SDF-1 in the peri-infarct period would lead to improvements in left ventricular function.

We chose to use MSC to deliver SDF-1 to the infarct zone because they are easy to expand in culture, may be able to differentiate into cardiac myocytes (3, 24), and home to the newly infracted myocardium (25). We chose to use a cell therapy-based approach for the delivery of SDF-1 in order to induce a sustained release of SDF-1 similar to that which may be achieved through the transplantation of stem cells into the myocardium. Many recent studies suggest that some populations of MSC do express CXCR4 (26, 27); however, the extent to which CXCR4-expressing MSC home SDF-1 *in vivo* remains unclear. Inhibition of SDF-1:CXCR4 binding has only been shown to partially block recruitment of these MSC to the bone marrow (26). Also, MSC make SDF-1 (Fig. 1*a*), and there is little precedent for a cell that expresses both receptor and ligand to home to that ligand. Finally, the MSC delivery strategy employed in these studies is a noninvasive way to deliver genes to the recently injured heart. CXCR4-expressing MSC do respond to SDF-1. Consistent with our data, it was recently shown that SDF-1 leads to increased survival and growth of CXCR4-expressing MSC (18).

The engraftment of SDF-1-expressing MSC had multiple positive effects. Cardiac myocytes and muscle progenitor cells have been shown to express CXCR4 (28, 29). First, we found that cardiac myocytes naturally begin to express CXCR4 between 24 and 48 h after AMI (Fig. 3*a*). This observation suggests that delivering SDF-1 to the cell surface of injured cardiac myocytes could lead to inhibition of myocyte apoptosis as it did to MSC cultured under ischemic conditions (Fig. 2*a*). We observed an ~80% decrease in cardiac myocytes apoptosis at the infarct border zone in those animals that received SDF-1-overexpressing MSC. This led to a significant increase in the survival of cardiac myocytes bundles within the infarct zone of those animals that received SDF-1-expressing MSC.

Second, the overexpression of SDF-1 in the infarct zone resulted in neovascularization. This is likely due to the increased recruitment of endothelial progenitor cells, as we have shown in a model of ischemic cardiomyopathy (14). There was no gross pathological evidence of hemangioma formation from the sustained expression of SDF-1 over 5 wk (30)

Third, the overexpression of SDF-1 in the infarct zone unexpectedly led to a marked increase in the number of smooth muscle α-actin and connexin 45-expressing cells that appear to form a band along the middle of the infarct zone. While some of these cells are from the MSC that were infused 1 day post-MI, the majority are not. Furthermore, most of these smooth

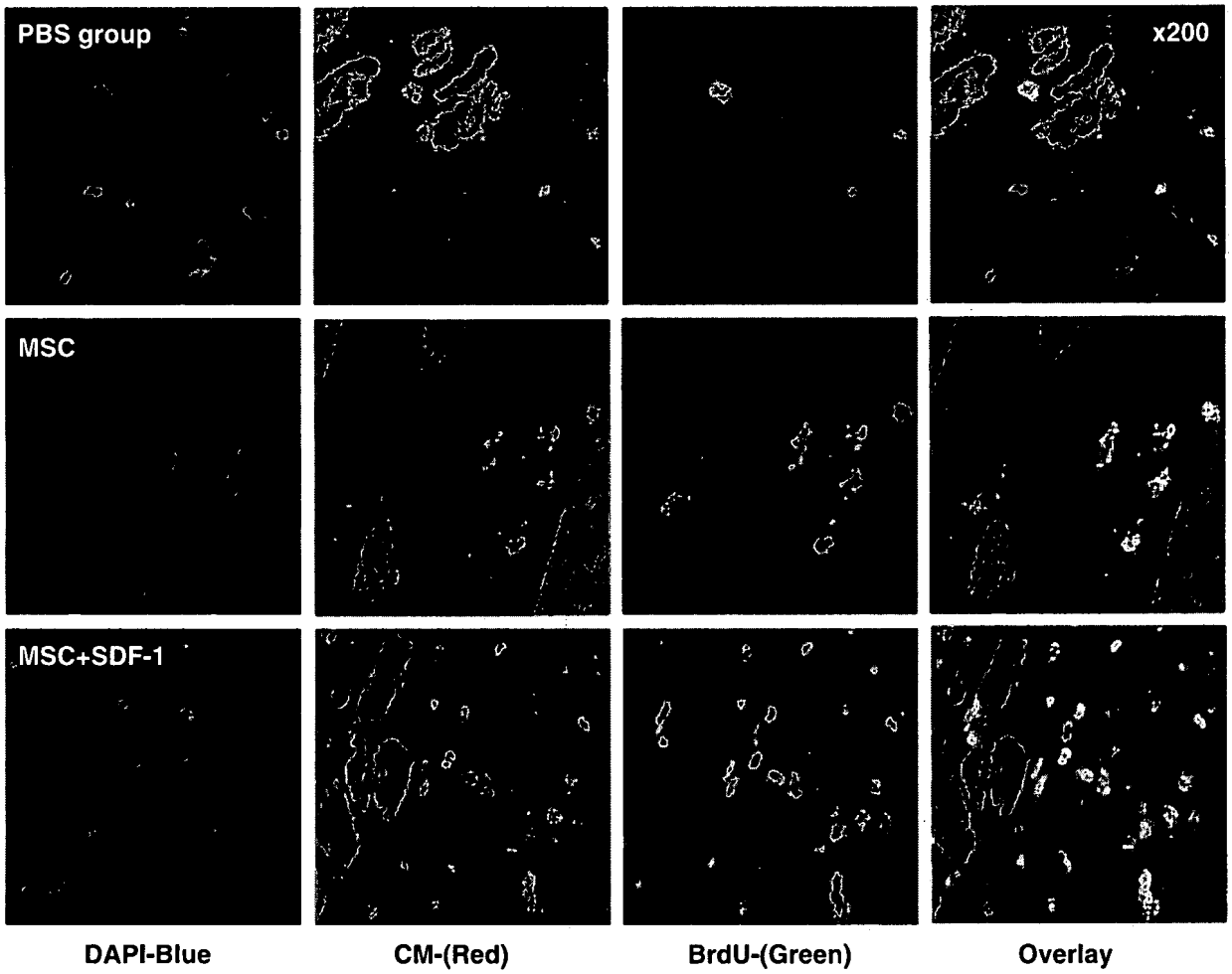

Figure 6. All representative images are from tissue 5 wk after AMI and infusion of 2 million of control or SDF-1-expressing GFP-positive MSC 1 day after AMI. All animals received BrdU twice daily for 14 days beginning on the day after cell transplantation. Confocal images of immunofluorescent staining in the infarct border zone for cardiac myosin (red), BrdU (green), and cell nuclei (DAPI, blue) from animals that received PBS or control or SDF-1-expressing MSC. Column of images on the right are the overlay in which red and green overlay is depicted as yellow.

muscle cells were not associated with blood vessels, as demonstrated by a void of vWF or connexin 40 expression in the area of the SMC. While it is not clear that these cells contract in unison, it is intriguing to note that these SMC express connexin 45 and may contract in response to mechanical stretch during the cardiac cycle.

Route of delivery

Studies have sought to define the ideal route of cell delivery, including mobilization from bone marrow (7, 31), catheter-based intracoronary infusion (4, 32), and intramyocardial injection (1). Catheter-based intracoronary delivery of MSC in the left circumflex artery of dogs led to microinfarction (33), which may not be well tolerated in patients with little cardiac reserve. Our results highlight the fact that a simple intravenous infusion may be highly effective, while at the same time minimize mechanical risk to the freshly injured myocardium. This may be due to the fact that the intravenous infusion of cells results in dilution of the cells prior to entry into the coronary vasculature as well as filtering of cell clumps in the lung.

MSC differentiation

Reports in the literature suggest that MSC delivered during the peri-infarct period can differentiate into cardiac myosin-expressing cells. Despite being able to significantly increase MSC survival in post-MI myocardium, MSC labeled with BrdU or GFP did not demonstrate significant regeneration of cells with a cardiac myocyte phenotype. Our study suggests that the infused MSC differentiate into myofibroblasts that express connexin 45, which is consistent with our recent observations that MSC delivery at the time of myocardial infarction leads to improved electrical impulse conduction into the scar and a decrease in the ability to induce ventricular tachycardia (34). While it is possible that a small population of the engrafted MSC may have differentiated into a cardiac

myocyte phenotype, our data are consistent with the hypothesis that the overall benefit of MSC therapy is not due to regeneration, but rather preservation of cardiac tissue, and that at least one factor mediating this effect is SDF-1.

CONCLUSIONS

Our data are consistent with the concept that there is a naturally occurring regenerative repair process that occurs in infarcted myocardium that can be enhanced through the overexpression of SDF-1 within the myocardium after myocardial infarction. We observed multiple beneficial effects on the myocardium, apparently independent of the effects of the intravenously delivered stem cells themselves. Rather, these observed beneficial effects may be due to local paracrine effects, and could explain the improvement in cardiac function observed with the introduction of unfractionated bone marrow preparations in the peri-infarct period. These studies demonstrate that stem cell transplantation may have significant effects on cardiac function independent of cardiac myocyte regeneration and that strategies designed to exploit these effects may lead to significant preservation of cardiac function. Several studies have demonstrated the utility and safety of allogeneic and autologous MSC infusion in clinical populations (35, 36); thus, translation of an SDF-1-based therapy for preservation of myocardial tissue to patients with acute myocardial infarction should be possible.

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